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Real-Time PCR: Understanding C_T

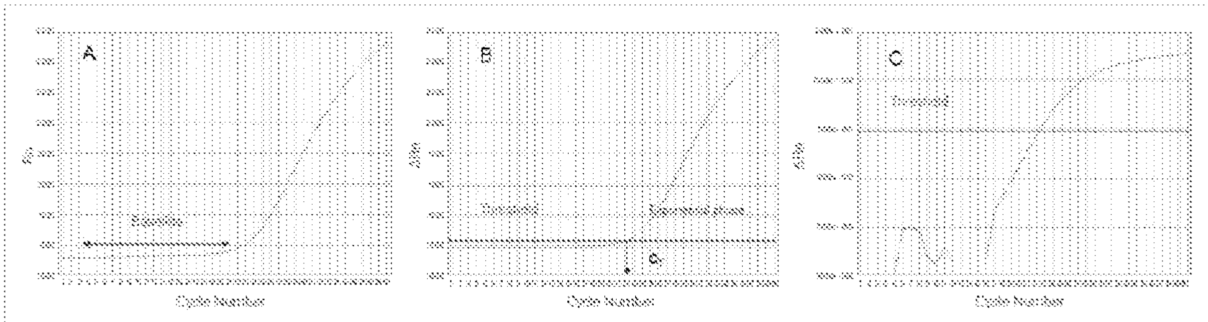

Figure 1. A: Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. In other words, Rn is the reporter signal normalized to the fluorescence signal of ROX™. In this view, Rn is graphed versus cycle. B: ΔRn is Rn minus the baseline, graphed here versus the cycle of PCR. C: Amplification plot shows the Log ΔRn graphed versus cycle.

Introduction

Real-time PCR, also called quantitative PCR or qPCR, can provide a simple and elegant method for determining the amount of a target sequence or gene that is present in a sample. Its very simplicity can sometimes lead to problems of overlooking some of the critical factors that make it work. This review will highlight these factors that must be considered when setting up and evaluating a real-time PCR reaction.

Factors that can Influence C_T

C_T (threshold cycle) is the intersection between an amplification curve and a threshold line (Figure 1B). It is a relative measure of the concentration of target in the PCR reaction. Many factors impact the absolute value of C_T besides the concentration of the target. We will discuss the most common template-independent factors that can influence C_T and describe how to evaluate the performance of a real-time PCR reaction.

Figure 1 shows several parameters of the real-time reaction amplification plot. The exponential phase in Figure 1B corresponds to the linear phase in Figure 1C. The threshold must be set in the linear phase of the amplification plot in Figure 1C. The C_T value increases with a decreasing amount of template. However, anything from the reaction mix or instrument that changes the fluorescent measurements associated with the C_T calculation will result in template-independent changes to the C_T value. Therefore, the C_T values from PCR reactions run under different conditions or with different reagents cannot be compared directly.

The Effect of Master Mix Components

The fluorescent emission of any molecule is dependent on environmental factors such as the pH of a solution and salt concentration. Figure 2 shows the raw fluorescence data of a TaqMan® probe in the background of two different master mixes. Note that the fluorescence intensity is higher in Master Mix A even though the target, probe and ROX™ concentrations are the same in both cases.

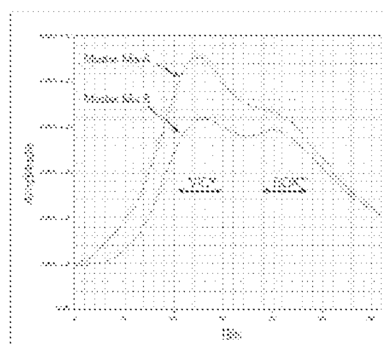

Figure 2. Raw fluorescence data obtained with one assay and two master mixes with the same ROX™ level. The difference in signal is due to the master mix composition. Reaction was performed on an Applied Biosystems 7900HT Fast Real-Time PCR System with a VIC® MGB probe. The X axis shows the emission wavelength of the fluorophore and the Y axis shows the intensity of the emission.

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The resulting ΔRn value will, therefore, vary as shown in Figure 3. Note that the baseline fluorescence signals, in a template-independent factor, are different for the two master mixes (Figure 3A). Variations in C_T value do not reflect the overall performance of the reaction system (Figure 3B). Master mixes with equivalent sensitivity may have different absolute C_T values.

ROX™ Passive Reference Dye

The Rn value is calculated as the ratio of FAM™ fluorescence divided by the ROX fluorescence. Therefore, a lower amount of ROX would produce a higher Rn value assuming FAM fluorescence signal stays the same. This would lead to an increase in baseline Rn and subsequently a smaller ΔRn as well as a different C_T value. The different C_T value obtained by lowering the ROX level has no bearing on the true sensitivity of the reaction, but can have other unintended consequences. Low ROX concentration can result in increased standard deviation of the C_T value, as shown in Figure 4. The greater the standard deviation, the lower the confidence in distinguishing between small differences in target concentration (see the precision section on the next page).

Efficiency of a PCR Reaction

The efficiency of a PCR reaction can also affect C_T. A dilution series amplified under low efficiency conditions could yield a standard curve with a different slope than one amplified under high efficiency conditions. In Figure 5, two samples (X and Y) amplified under low and high efficiency conditions show different C_T values for the same target concentration. In this example, although the high efficiency condition (the blue curve in Figure 5) gives a later C_T at high concentration, it gives better sensitivity at low target concentration.

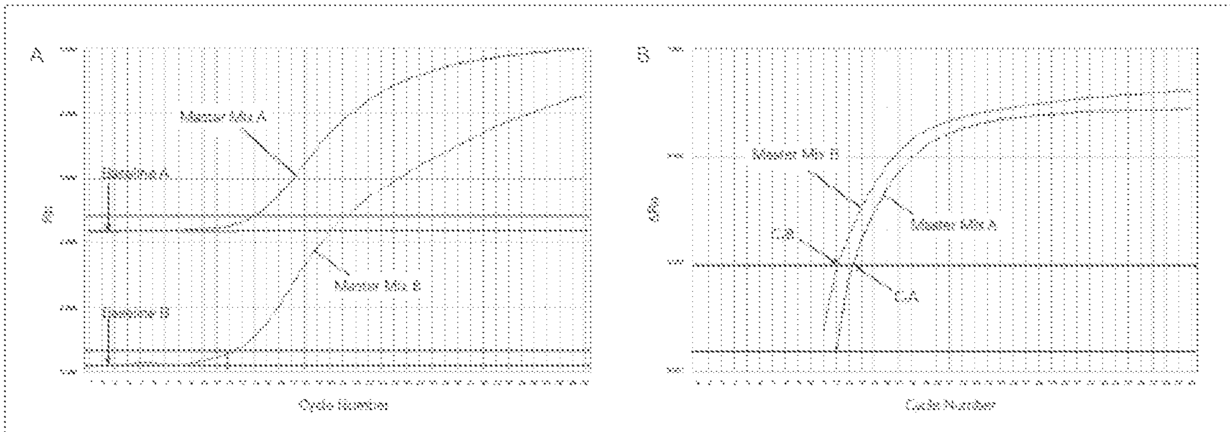

Figure 3. Master Mix A and Master Mix B were used to amplify RNase P in equal amounts of human gDNA using the Applied Biosystems 7900 Real-Time PCR System. Figure 3A shows the Rn versus cycle number and the baselines for both reactions. Figure 3B shows the Log (ΔRn) versus cycle number. The threshold (green) is set at the same level for both master mixes. The C_T value of Master Mix B (C_TB) is earlier than that of Master Mix A (C_TA) for identical concentrations of target, reflecting the lower baseline of Master Mix B.

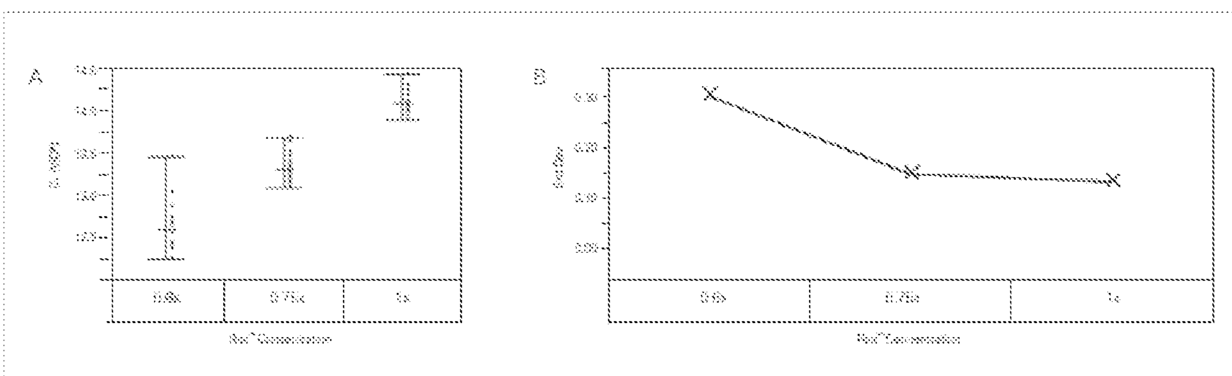

Figure 4. Master mixes containing 3 different concentrations of ROX™ were used to amplify the TGF beta assay on the Applied Biosystems 7900HT Fast Real-Time PCR System using the 96-well block. Figure 4A shows the C_T value and Figure 4B shows the standard deviation with variable ROX concentrations. Decreasing ROX concentration gives an earlier C_T but increases the standard deviation.

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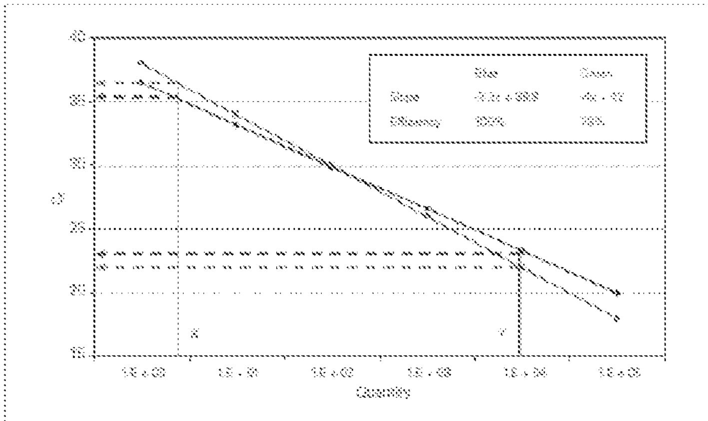

Figure 5. The blue standard curve has an efficiency of 100% (slope is -3.3). The green standard curve has an efficiency of 75% (slope is -4). Amplification of the Y quantity gives an earlier C_T with low efficiency condition (green) compared to the high efficiency condition (blue). With a lower quantity (X) there is an inversion and the low efficiency condition (green) gives a later C_T compared to the high efficiency condition (blue).

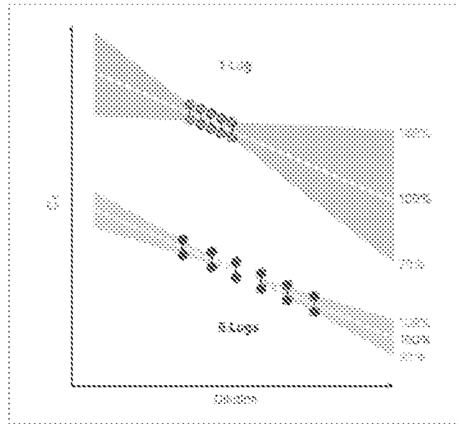

Figure 6. Accurate calculation of PCR efficiency depends on the range of template amount used for the serial dilution used to calculate the efficiency. With a 2-fold dilution with 5 points (orange), the potential artifact is higher than for the 10-fold dilution with 5 points (blue).

The PCR efficiency is dependent on the assay, the master mix performance and sample quality. Generally speaking, an efficiency between 90-110% is considered acceptable.

The observation that the C_T value produced from one sample is higher than that of the other, could be valuable in concluding that the amount of template is less in the first sample, assuming all other factors such as instruments, reagents and assays are equal. However this is not true if different instruments, reagents, primers and probes or reaction volumes are involved in producing the two C_Ts. Therefore, the absolute C_T value comparison is only meaningful when comparing experiments using the same reaction conditions as defined above.

How to Evaluate the Performance of a Real-Time PCR Reaction

In order to compare two reactions where a condition is changed (for example two different master mixes or two different instruments), the following parameters must be evaluated.

Dynamic Range

To properly evaluate PCR efficiency, a minimum of 3 replicates and a minimum of 5 logs of template concentration are necessary. The reason for this suggested level of rigor is illustrated in Figure 6, which demonstrates the possible mathematical variation of slope/efficiency one gets when testing dilutions over 1 log vs. 5 logs. Thus, even if the assay is 100% efficient, one can get a range from 70-170% when testing a dilution series of a single log, due to the standard deviation in one dilution. Doing the same number of dilutions/replicates on a 5-log range, the potential artifact is only +/- 8 %. That means that if we find 94% efficiency on a 5-log range, the assay would have a range of 88% to 100% efficiency. To accurately determine the efficiency of a PCR reaction, a 5-log dilution series must be performed. A slope of -3.3 +/- 10% reflects an efficiency of 100% +/- 10%. A PCR reaction with lower efficiency will have lower sensitivity.

R^2 Value

Another critical parameter to evaluating PCR efficiency is R^2, which is a statistical term that says how good one value is at predicting another. If R^2 is 1 then you can perfectly predict the value of X (quantity) with the value of Y (C_T) (Figure 7A). If R^2 is 0, then you cannot predict the value of X with the value of Y (Figure 7B). An R^2 value >0.99 provides good confidence in correlating two values.

Precision

The standard deviation (square root of the variance) is the most common measure of precision. If many data points are close to the mean, the standard deviation is small; if many data points are far from the mean, the standard deviation is large.

In practice, a data set with a sufficient number of replicates forms an approximately normal distribution. This is frequently justified by the classic central limit theorem which states that sums of many independent, identically-distributed random variables tend towards the normal distribution as a limit. As shown in Figure 8A, about 68% of the values are within 1 standard deviation of the mean, about 95% of the values are within two standard deviations, and about 99.7% lie within 3 standard deviations.

If a PCR is 100% efficient, there is one C_T between the mean of a 2-fold dilution (Figure 8B). To be able to quantify a 2-fold dilution in more than 99.7% of cases, the standard deviation has to be ≤ 0.167. The greater the standard deviation, the lower the ability to distinguish between 2-fold dilutions. To be able to discriminate between a 2-fold dilution in more than 95% of

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cases, the standard deviation has to be ≤ 0.250 (Figure 8C).

Sensitivity

Any system capable of effectively amplifying and detecting one copy of starting template has achieved the ultimate level of sensitivity, regardless of the absolute value of the C_T.

As described earlier, efficiency is a key factor in determining the sensitivity of a reaction (Figure 5). Another important consideration with detecting very low copy numbers is that the distribution of template would not be expected to be normal. Instead, it would follow a Poisson distribution which predicts that in a large number of replicates containing an average of one copy of starting template, about 37% should actually have no copies, only about 37% should contain one copy, and about 18% should contain two copies (see Figure 9). Thus, for a reliable low copy detection, a large number of replicates are necessary to provide statistical significance and to overcome the Poisson distribution limitation.

Conclusion

These factors – efficiency, R^2, precision, sensitivity – are used to determine performance of a PCR reaction when comparing different reaction conditions. For a rigorous evaluation, all factors listed in Table 1 must be evaluated together.

In addition to these factors, proper experimental controls (such as no template control, no RT control) and template quality must be evaluated and validated.

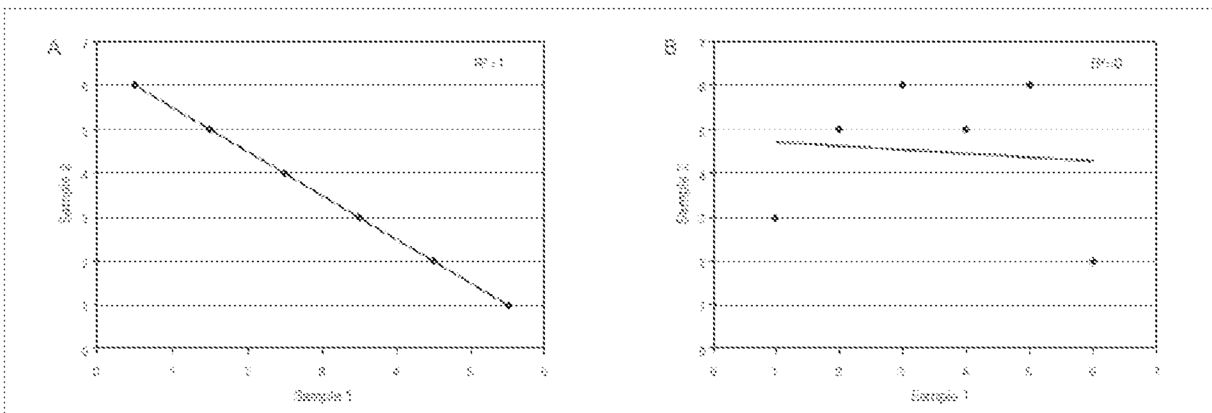

Figure 7. Example of R^2 value calculated for 2 straight lines. A. There is a direct relation between x and y values. B. There is no relation between x and y values.

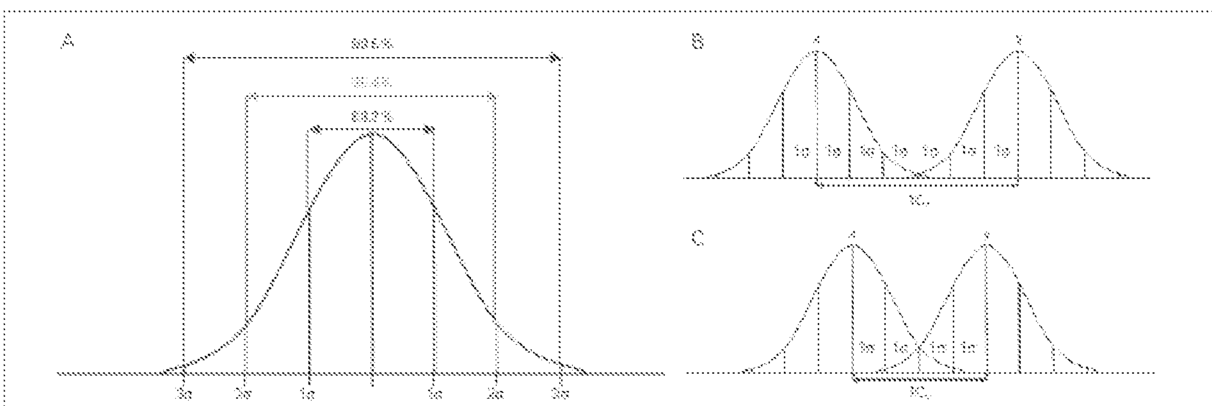

Figure 8. Normal distribution and standard deviation. (A) shows a normal distribution of data. If the PCR efficiency is 100% there is one C_T between the mean of 2-fold dilution samples (sample X and sample Y). To be able to quantify both samples in 99.7% of cases, the standard deviation has to be less than 1 C_T divided by 6 standard deviations (1/6=0.167), shown in (B). To be able to quantify both samples in 95% of the case, the standard deviation has to be less than 1 C_T divided by 4 standard deviations (1/4=0.25), shown in (C).

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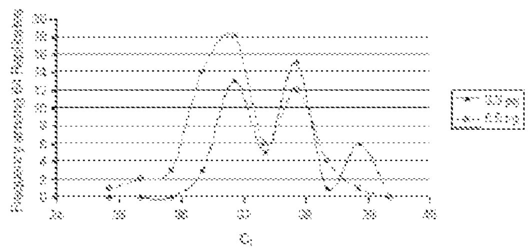

Figure 9. Poisson distribution for low copy number. The blue curve represents Poisson distribution for 3.3 pg of DNA (1 copy of DNA). The pink curve represents Poisson distribution for 6.6 pg of DNA (1 cell, 2 copies of DNA).

Factors	Recommendations	Criteria
Efficiency	Serial dilution with 5-log dilutions	Slope= -3.3
		$R^2 > 0.99$
Precision	Minimum of 3 replicates	Standard deviation < 0.167
Sensitivity	High replicate number of reactions for low copy number sample input due to Poisson distribution	Statistical test analysis

Amplification Plot
An amplification plot is the plot of fluorescence signal versus cycle number. Reactions are characterized by the point in time during cycling when amplification of a PCR product is first detected. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

Baseline
In the initial cycles of PCR there is little change in fluorescence signal. This defines the baseline for the amplification plot. In these cycles we see the fluorescence background of the reaction. This will be subtracted from the results when setting the baseline. (For information of how to set up the baseline, download the document "Applied Biosystems 7300/7500 Real-Time PCR System" PN 4347825 from the Applied Biosystems website **www.appliedbiosystems.com**)

Delta Rn
ΔRn is the normalization of the Rn obtained by subtracting the baseline (ΔRn = Rn - baseline).

Passive Reference
A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations caused by change in concentration, volume or sample effects.

PCR Efficiency
The equations below describe the exponential amplification of PCR.

$Cn = Ci * (1 + E)^n$
Ci = initial copy number
Cn = copy number at cycle n
n = number of cycles
E = efficiency of target amplification

If efficiency is maximum (=1) the equation is: $Cn=Ci * 2^n$ and it means that the fold increase will be 2 at each cycle. If the efficiency decreases, the quantity of PCR product generated at each cycle will decrease and the amplification plot will be delayed. The recommended efficiency is between 90 to 110%.

Reporter Dye
Reporter dye is the dye attached to the 5' end of the TaqMan® probe. The dye provides a fluorescence signal that indicates specific amplification. If SYBR® Green I is used, this dye binds double-stranded DNA and the increase of fluorescence signal indicates amplification as well. Specificity should be checked with a melt curve (*Power SYBR® Green PCR Master Mix and RT-PCR Protocol, P/N 4367218*) or gel analysis of the PCR product.

Rn
Normalized reporter is the ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye.

Threshold
A level of ΔRn used for the C_T determination in real-time assays. The level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the amplification plot defines the C_T (threshold cycle.) For information on how to set up the threshold, download the document "Applied Biosystems 7300/7500 Real-Time PCR System" PN 4347825 from the Applied Biosystems website **www.appliedbiosystems.com**

Threshold Cycle (C_T)
The fractional cycle number at which the fluorescence passes the threshold.